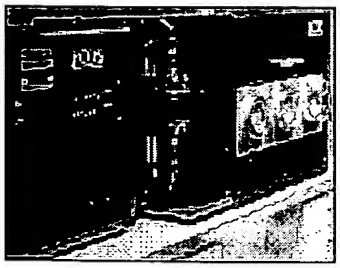

Large Scale Oligonucleotide Synthesizers

I. Basic Principles and System Design

Preliminary Draft

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ABSTRACT

The central problem in scaling up oligonucleotide synthesis is to expose each element of a large bed to the same conditions obtaining in very small ones, for the same intervals of time. Our analysis suggests that scale up is chiefly limited by fluid path length through the bed. By using annular beds in zonal centrifuge rotors of unique design, with fluid flow controlled by combining centrifugal force with differences in physical density between reagents, reagent fronts may be kept exactly perpendicular to the direction of flow in each bed element. Under these conditions bed volume may be increased by increasing rotor length and diameter. The rotor is lined with polypropylene or Teflon®, and has a thick tempered glass end-window. Transparent rotary valves of a unique design allow any of 47 reagents to be selected, and the direction of flow through the rotor controlled. A photodiode spectrophotometer provides complete absorption spectra on fluid in the rotor inlet and outlet lines every four seconds, and a large balance weighs effluent from the synthesizer continuously. The entire operation is controlled by a work station, and steps may be programmed by time, absorbance, or reagent mass. Reagents are identified by spectra, and trityls are integrated on line. A detailed time-stamped log file provides a complete record of each synthesis.

Key Words: Oligonucleotide synthesis, antisense, phosphorothioates, specific sequence drugs, aptamers, ribozymes.

INTRODUCTION

This paper describes a new class of biopolymer synthesizers in which synthesis occurs in an annular bed of solid phase support in a zonal centrifuge rotor. A combination of centrifugal force and density differences between solutions is used to quantitatively displace one solution by another. The result is an efficient synthesis system, with decreased reagent utilization, which can be scaled up to production levels.

Ordered-sequence biopolymers, including conventional DNA, antisense DNA, RNAs including ribozymes and nexamers, peptides, so-called peptide DNAs, aptamers, diversomers, polysaccharides, and a variety of new compounds composed of ordered arrays of monomers not normally found in biological systems (diversomers), now hold great promise as pharmaceuticals (1). The alternatives for manufacture of these compounds include stepwise solution phase synthesis, stepwise solid phase

synthesis, and fragment assembly either in solution or on solid phase supports. For the near future it appears that compounds longer than approximately ten monomers (10-mers) will be synthesized using solid phase methods based on protocols which are currently available. While this paper is concerned with oligonucleotide synthesis, many of the problems considered relate to the synthesis of all types of ordered polymers.

Thus far, no one has reported the development of oligonucleotide synthesizers capable of producing the batch sizes which will be required if any one of the approximately forty compounds now under development were to be approved for production as a pharmaceutical. Estimates of the amounts ultimately required vary from hundreds of grams per year for some topical applications, to metric tons per year for some systemic uses. Requirements will also vary depending on whether the application is for AIDS, tropical parasites, a major human cancer, or for a rare disease afflicting only a few individuals, and also on the dose required per patient.

The problem of developing production synthesizers is rendered more complex by the FDA requirement that production systems and methods be in place when a new drug is approved, i.e., it is necessary to show that the drug can indeed be produced. After approval it is difficult and costly to change methodologies. While amounts of drug sufficient for animal and some initial clinical trials can be made using present synthesizers, companies aiming to develop and produce new classes of sequence-specific drugs are faced with the problem of concurrently developing both the drugs and new production- scale systems for their synthesis.

It might be thought that synthesis can be scaled up simply and linearly by increasing the size of each component, and of reagent volumes. In practice, however, it appears to be a universal experience that the efficiency of oligo synthesis drops off as synthesis is scaled up, for reasons which have not been fully understood. Unfortunately, unsuccessful results are either not reported, or not reported in detail. Hence it is difficult to assemble and inter-compare data and experience on scaling up oligo synthesis from different laboratories, especially when proprietary sequences and methods are involved. We have been unable to find published data on synthesis in beds having volumes in the range of 300 ml to several liters. We are, however, aware of laboratories continuously running ten or more conventional commercial instruments producing a few grams of crude product per run, and pooling these batches for animal and clinical studies. It is difficult to escape the conclusion that a requirement is emerging for much larger scale synthesizers.

Although considerable advances have been made in solution phase synthesis of oligonucleotides (2), it appears that for the near term large scale oligo synthesis will be done on solid phase supports (3). We therefore consider here only problems relating to the scale up of solid phase synthesis, and demonstrate that synthesis in a zonal centrifuge allows precise control of fluid flow in large beds having a short path length.

SCALE UP OF SOLID PHASE OLIGONUCLEOTIDE SYNTHESIS

Scale up problems in oligonucleotide (oligo) synthesis arise partly from the very high cost of doing extended large scale optimization studies, and, more importantly, from factors which appear to be inherent in the physical chemistry of scale up itself. Present small- and intermediate-scale protocols have been optimized during thousands of runs in many laboratories and involve many chemical tradeoffs, often between reagent concentration and time. The conventional cycle involves use of a solid phase support with the first nucleoside, plus protective groups, already attached. After washing the support, the terminal trityl group is removed (deblocked) in acid in dichloromethane (DCM), and the deblocking solution replaced with dry acetonitrile (ACN). The first synthon (usually a phosphoramidite) plus an activator (tetrazole) is added to couple the second protected nucleotide. Coupling time is adjusted to achieve maximum coupling efficiency. If coupling time is prolonged, some detritylation may occur, resulting in double coupling, and may also result in the formation of

branched chains, especially at a guanine residue. After additional washing in acetonitrile, the phosphorus is oxidized in a partially aqueous iodine solution if a conventional DNA oligo is being synthesized, or sulfurized (for example with the Beaucage reagent) if a phosphorothioate is being made. After additional washing, a capping solution is introduced to cap unreacted nucleotides. This step is followed by additional washing in preparation for the next repeating cycle.

Deblocking to remove trityl groups is a reversible reaction which must be carried as close to completion as possible. The acid used must be strong enough to remove these groups and wash them away, but not strong enough to cleave the N-C1' glycosidic bond in purine deoxy- nucleosides to produce depurination. If the trityl groups are not completely removed, or if they are allowed to recombine, the next coupling step will not extend those trityl-protected nucleotides, resulting in a "deletion mutation". Also, we have discovered that as the oligonucleotide being synthesized is lengthened, trityl groups tend to adsorb and be retarded chromatographically in the CPG support. This effect appears to be due to the fact that the growing oligo chains impart ion exchange characteristics to the support. Hence it may be necessary to change washing conditions through a synthesis.

Coupling of phosphoramidites to the growing chain can only be done in the absence of water. However, water is often included in the oxidation step, and must be removed by extensive and complete washing before the next coupling step.

Final yield is a function of coupling efficiency, and efficiencies of 99% or over are sought. As has been noted (4), "none of these (reactions) can be regarded as totally safe", and, "the essential principle remains that a slight change in reaction conditions ... can give a very large change in the amount of side product." Problems in scaling up DNA synthesis recur in RNA scale up but with the added difficulty that additional protecting groups are employed.

We conclude that there are two alternative strategies for developing large scale solid phase oligonucleotide synthesizers (and later peptide and polysaccharide synthesizers). The first is to use large conventional reactors or columns, and to attempt to alter and improve the chemistry through a series of systematic and very expensive studies (which may not be successful). It will be difficult, however, to escape the basic problem of conventional linear scale up which is that it becomes increasingly difficult to produce rapid and complete solvent and reagent changes in the times allotted, and, as the size of the system is increased, to treat all elements of the bed identically. Solid phase synthesis may appear superficially to be analogous to chromatography, however chromatography rarely deals with time dependent chemical transformations, or with the problem of minimizing the usage of expensive solvents and reagents through a long series of repeating cycles.

The second approach is to use the reagents and schedules which are successful on a small scale, and attempt to devise a new reactor which duplicates small-scale conditions for all individual solid phase support particles in a bed, exposing each to exactly the same conditions for the same intervals of time, and to achieve these conditions by using centrifugal force combined with density differences between solutions to control flow through an annular bed. We have adopted the latter approach.

There is an additional advantage to the second approach which is that it allows new protocols which have been developed on small or intermediate-scale systems to be directly translated to production synthesizers. Protocols used for one can run on the other, for the simple reason that the bed path length can, as will be shown, remain comparable.

Development of a Monitoring and Reagent Delivery System:

A wide variety of configurations have been employed to valve a series of different solutions into a reaction vessel, and to monitor some aspects of the synthesis process - all under computer control. The requirement here is for a design which includes:

1. Reagent sequencing valves which can be scaled up both in terms of flow-through and the number of inlet ports;
2. A reversing, purging, and drain valve which can reverse flow through a centrifugal reactor or can purge all lines to drain;
3. Positive means for producing fluid flow;
4. A spectrophotometer which continuously monitors the optical absorbance over a wide range of wavelengths of both input and output streams;
5. Means for measuring flow through the system continuously; and
6. A work station which controls the entire system, monitors all variables, displays results rapidly, and provides an complete and detailed record of each synthesis.

In subsequent papers we will describe the use of this system to evaluate different protocols and different rotor sizes. The system is shown diagrammatically in Figure 1.
We have decided at the outset to make the system, including computer control and programming, literally and figuratively as transparent as possible so that all operations may be either inspected visually, or may be monitored through instruments with the results conveniently displayed in real time. Reagents contact a limited range of resistant materials (Teflon®, glass, polypropylene, Kalrez®, zirconia, quartz, or sapphire), and use of metal (to prevent any possible contamination of the product by heavy metals) has been avoided in initial designs.

Fluid Transport:
A versatile system is required for producing a wide range of flow rates against differing resistances. We have examined pumps, gravity flow, and gas pressure. Use of pumps requires that there be a positive method for detecting overpressures and for stopping them if a valve is inadvertently closed, or if a line is clogged. Further, pumps of a wide range of sizes would be required as the system is scaled up. Materials of construction are presently limited to fluorocarbons including Kalrez®, glass and some other ceramics, thus restricting the range of available pumps considerably. Gravity flow may ultimately be the best choice, with fluids of different density arranged at different heights to achieve uniform flow. For 10 psi the reagent reservoirs need only be an average of 20 feet above the valves - a situation achievable in a production environment. For the present experimental test-bed system, however, gas pressure appears the best choice using dry helium because of its low solubility. In the present system we have used large plastic-coated reagent bottles, and closures of our own design.

Valving:
An enlarged set of reagents is required for the synthesis of highly modified sequences. These may include, for example, use of new polymer backbones, sulfurization of only a few phosphates on an antisense DNA, use of new and unique monomers, and the addition of end groups which promote transport into cells, or which target specific cell types. In addition, when physical density is used to control flow through a bed, wash solutions of intermediate densities may be required, thereby increasing the number of reagents used. When gas flow cannot be used to purge a system all the way to and through a reaction bed, as is the case with centrifugal systems, it is advantageous to used multiport valves to replace the rows of solenoid valves which are conventionally gas-purged.
The zero-hold-up valves we have developed are shown diagrammatically in Figure 2 and Figure 3. The valves are designed on the theory that all valves leak, even though the leakage may be too small to detect, and that the operation of the valves and any leakage should be visible to the operator. The valves therefore have transparent faces. The reagent valves consist of a stationary valve block with 24 input lines arranged in a circle, and one output line at the center. The face of the valve is a thick plate of Pyrex® or quartz, which contains an embedded channel connecting the center line to one of the edge lines, as is shown in Figure 6. The plate is supported on a precision bearing, and is rotated by a

computer-programmed stepper motor. The stationary block is fabricated from glass-filled Teflon®. The rotating plate is ground flat to within 1/2 wavelength of light. The stationary valve block is undercut leaving three rings in contact with the face plate. These are the center exit line ring, the ring including the 24 input lines, and an additional ring at the edge so that any leakage is caught and is visible, thus giving two concentric chambers which are additionally connected with small holes so that fluid or air can drain from the inner chamber to the outer one. Two additional lines connecting to the top and bottom of the outer chamber allow acetonitrile or other solvent to be introduced into the bottom of the valve face and out the top, and also to drain back when the input line is connected to drain, thus washing away any solutes which might crystallize at the edges of the contact areas.
The lower valve (Valve 2) in Figures 1 and 3 connects to, and drains into Valve 1, giving a maximum of 47 reagent input lines. Additional reagent valves may be added if a larger number of reagents is required.
The input lines are arranged so that all repeating reagents, except activators to be mixed with synthons, are programmed through valve 1, and the reagents of valve 1 are in the order used. Synthons are introduced through valve 2, and synthons and activator lines are present in pairs interspersed with solvent lines. At the synthon coupling step, line 1 of valve 1 is connected to the center line draining valve 2. It is evident that as the face plate revolves over each synthon line and each activator line during a full rotation, a very small amount of synthon solution is introduced into the channel in the face plate and carried along. The valve is therefore stopped briefly at each solvent line (between the synthon and activator pairs) to wash out the line to drain whenever the valve is moved. Each synthon is mixed with activator by oscillating the valve between the two adjacent positions to give a mixture containing equal volumes of synthon and activator. Tests with colored solvents demonstrated the complete mixing of two components before entering the rotor.
The third valve is a reversing and drain valve which connects the output from valve 1 to either the top or the bottom of the rotor through flow cells in the spectrophotometer, or to drain. A close view of the rotating face plate with dye in the line is shown in Figure 4.

Absorbance Measurements:
Absorption spectra of fluid flowing through the input and the exit lines are recorded from 190 to 800 nm using a Hewlett-Packard Model 8452A photodiode array spectrophotometer. The initial blank is taken using acetonitrile in the case of oligonucleotides, and spectra are recorded from 0 to 3 optical density units during an entire run. The double flow cell with quartz windows was constructed using a Kalrez® gasket to give flow paths of 0.8 mm and 0.78 mm for the input and exit lines respectively, as determined using a dye solution of known optical density. The flow cell is moved using a computer-controlled gas-pressure-driven movement at two second intervals to give one complete spectrum from each line every four seconds. During deblocking, the input flow cell is not monitored, and the exit flow cell is monitored at one second intervals to obtain more data points. Absorbance data can be displayed in real time as discussed below, and all absorbance spectra from an entire run are also stored for subsequent evaluation.

Flow Measurements:
In large scale systems it is essential to measure flow through the system continually as the final check for possible valve failure and leaks, and to insure that the desired amount of reagent is delivered at each step. Flow measurement also provides one option for programming, and valve movements may be based on either time or integrated flow for each step. A variety of flow meters are available, and the best of these uses intermittent pulse heating of a stream, with downstream detection of each pulse. However, the most robust means for measuring flow is to continuously weigh the effluent, and multiply the weights by the physical densities of the solutions used. We have incorporated into the

present system an electronic balance capable of measuring weights up to 161 kg with one-gram precision (Sartorius Model F150S-D2).

Physical Density Measurements:
The physical densities (gm/cc) of the solutions used are measured as prepared using a Mettler DA-110 density/specific gravity meter, which measures to four decimal places. We have also incorporated these into the synthesizer to measure density on-line. However, present versions of these instruments were found to be fragile and unreliable in prolonged use, and further development is needed to produce a routinely useful on-line device. Density measurements can be very useful however, and provide an additional check on the composition of reagents actually flowing in and out of the rotor.

ALTERNATIVE REACTION VESSEL CONFIGURATIONS

We have described a system which can deliver reagents in a programmed sequence, can monitor the synthesis as it progresses, and can be scaled up using larger versions of the same components, all using the same program and work station. The next question is that of choosing a reaction vessel configuration which can be similarly scaled up.
Five general preparative reaction vessel configurations have been proposed which are:

1. An enlarged packed column;
2. A fluidized bed with resuspension and mixing using gas;
3. A fluidized bed with suspension produced by mechanical shaking;
4. A zonal centrifuge system in which flow is stabilized by a combination of centrifugal force and density differences between solutions; and
5. A combination system which can combine two or more of the above.

We review these here to illustrate the problems besetting the first three, and then discuss how these are solved in a centrifugal system.

Scaling Up by Increasing Packed Column Volume:
Oligo synthesis in small columns works, even though the available solid phase synthesis supports are heterogeneous in size and give rise to small micro-differences in resistance to flow in the bed. While these small- scale flow anomalies are inconsequential in small columns, they produce problems in large columns, especially at fast flow rates.
In controlled-pore glass (CPG) the included volume is approximately 40% of the bed volume (5). Exchange between the included volume and the flowing excluded volume thus produces an additional widening of zones or fronts. The net effect is that particles at the top of a column experience sharp changes in concentration as each new reagent moves into the column and are exposed to the maximum reagent concentration, while particles at the bottom experience gradual and asynchronous changes at both the advancing and trailing boundaries. For very fast reactions, for example when activators which yield coupling times of 30 seconds or less are used, it will be desirable to run reagents through in narrow concentrated zones so that up to three different solutions may be in the bed at one time. If the zone is a narrow one, then the concentration in the zone center will diminish as it moves down the column, and the effect will be greater the longer the column. Zone or front broadening for any solid support is therefore a direct function of the column length. The effect of scaling up by merely lengthening a column is shown diagrammatically in Figure 5.
Hence it appears that the more ideal column is a relatively short one, with capacity gotten by increasing the cross sectional area of a column, with resultant difficulties in head space and the

collecting space below. Directing fluid in such a manner as to flow into the column with a sharp front perpendicular to the direction of flow over a large area is a difficult problem, and becomes more serious as the cross sectional area of the column is increased, and the anomalies shown in Figure 6 occur. Flow is more even when the bed is composed of homogeneous particles. Unfortunately no supports presently available are of uniform size and shape. The effects of imperfections in flow through a column may be diminished by repeatedly recirculating reagents through it; however this prevents the use of highly concentrated reagents (where the desired stoichiometry can be achieved in less than a bed volume), and recirculation does little to improve washing. However, if the actual synthon concentration in a column at one time is not sufficient to drive a coupling reaction to completion, it is useful to recirculate synthon through a column, or to introduce it in a series of steps of increasing concentration so that the final step drives the reaction as far toward completion as possible.

Synthesis Using a Gas-Stirred Fluidized Bed:

In a gas-stirred fluidized bed system the bed is resuspended and stirred using an inert gas such as argon or helium in a tapered vessel. The solvent may be removed by draining, with the external volume expelled by gas pressure to produce a semi-dry bed. Since the included volume is retained, that volume dilutes the next solution added. As a result, washing or any other change of solutions, no matter how many times repeated, approaches a quantitative replacement asymptotically, and many changes are required.

An advantage of this system is that gas pressure may be used to propel solutions, to wash reagents out of lines during solution changes, and to resuspend the bed. CPG is very sensitive to shear, and gas resuspension appears to be sufficiently gentle not to fragment the glass.

Synthesis Using a Mechanically-Stirred Fluidized Bed:- The synthesis chamber may also be mechanically shaken in a circular manner to fluidize the bed. The chamber configuration is usually different from that used for gas resuspension, and the shaking movement is controlled to prevent fracturing the support. In practice mechanical and gas resuspenson may be combined. The same disadvantages of included-volume carry over mentioned for gas-stirred vessels apply here also.

ZONAL SYNTHESIS ROTORS

While column and fluidized bed configurations have been successfully used for intermediate scale synthesis, for example in the range of 10 to 100 ml of bed volume, and could conceivably be scaled up further but with reduced efficiency, serious problems arise if kilogram and multi-kilogram batches of support are to be considered.

A solution to the fundamental problem is to carry out synthesis in specially designed zonal centrifuge rotors (6). Two problems are solved simultaneously. The first is to provide a configuration with a large bed volume and with a short path length, and the second is to use a combination of centrifugal force and density differences between solutions to provide very positive control of fluid flow through the bed.

The basic principles of zonal centrifuge rotor design and operation are well known (6), and hundreds of them have been built. In such hollow bowl rotors, flow is arranged to be along radial lines, with inflow from either the rotor center or the edge. Flow in the rotor is stabilized by either step or continuous density gradients, to produce circumferential isodense zones. These are quite insensitive to local differences in resistance to flow in a bed. Even if large void spaces exist in a bed in such a rotor, flow will be the same through the bed and the void. Flow is here stabilized in a manner unknown in chromatography at 1 x g. The basic concept is shown diagrammatically in Figure 7, which shows a small column of the type used in conventional synthesizers, a sector-shaped vessel of the same capacity, and the enlargement of the sector shape to annular beds of support having the same flow

path length, but with very much greater volumes. Flow through the annular bed in the zonal rotor is actually better controlled than in a small synthesis column because very positive and continuing forces are applied to the contained liquids. Hence the same schedule of solutions and the same time intervals can be made to apply to both. This is an enormous advantage in scaling up synthesis from a bench to a production level.

It is extraordinarily fortunate that the physical densities of the major solvents used are very different, being 1.325 g/mL for dichloromethane and 0.786 for acetonitrile at 200C. The maximum density difference to be exploited is therefore 0.539 g/mL. In a centrifugal field of only 100 x g, this translates into an effective density difference of 53.9 g/mL, or three times the difference in density between uranium metal and water. This provides both an extraordinary stabilizing force, and also a continuing force to move any stray liquid element rapidly to its proper density zone, even in the absence of bulk flow. This is quite different from the situation in a column at 1 x g where a wayward volume of fluid is only swept along by mass flow, and where density differences produce movement only very slowly. Actually the effects of density differences on flow through conventional packed chromatographic beds are rarely even considered in designing separations.

Note that the entire idea of using a centrifugal field is to stabilize zones. Bulk flow through the rotor is not due to centrifugal force, but to an applied external pressure. The familiar principles of the basket centrifuge therefore do not apply here. This is because the rotor is a closed system, and the two fluid lines employed to move fluid through the rotor are both on the axis of rotation.

The design used to spin the bed annulus shown in Figure 7 is illustrated diagrammatically in Figure 8 and the components of a synthesis rotor are shown in Figure 9. In practice, it is desirable to eliminate any contact between reagents and metals. Hence, as shown, the internal spaces are all lined with either glass. Teflon®, glass-filled Teflon®, or polypropylene. In the initial design, the rotating fluid-line seal is of glass-filled Teflon®, while the static seal is a polished glass hemisphere. The two fluid lines required are at opposite ends of the rotor to prevent cross contamination. Porous sintered polypropylene or Teflon® frits are used to keep the solid phase support in the rotor. So that packing of the solid phase support and movement of trityl-containing zones through the rotor can be observed, a tempered glass end window is included in the rotor.

In such a rotor, the fluid path through the bed can be short, with bed volume increased by increasing the average radius of the circumferential bed, by increasing rotor length, or both. Note that we have previously developed continuous-sample-flow-with-banding rotors with an annular volume of 3.6 L (8), and a total volume of approximately 8 liters, which are now operated at speeds up to 40,000 rpm. Very much larger rotors are feasible.

Unlike conventional zonal rotors, no vanes are required in synthesis rotors to prevent circumferential fluid movement due to Coriolis forces. The packed bed controls such flow. Care in designing the internal surfaces is required, however, to vector the circumferential iso-density zones into and out of the rotor. The methods by which this is done have been previously described in detail (6,7). We originally designed the rotor to hold a 400 ml bed volume, naively thinking that this would be considered a small volume, with subsequent rotors having multi-liter volumes. As we soon discovered, 400 ml is beyond the needs (or perhaps financial daring) of most antisense companies at this time. Three rotor cores with reduced volume were therefore designed which had capacities of approximately 18, 68, and 340 ml, the latter for flow studies. In these rotors the remainder of the volume is occupied by polypropylene.

Note that some organic solid phase supports swell and shrink during a synthesis cycle, giving rise to cracks in packed beds. These cause severe problems in non-centrifugal synthesis. However with density-gradient stabilized flow in a centrifugal field, such bed anomalies do not pose a problem since flow is almost completely immune to local flow restrictions. In addition, the centrifugal field itself contributes to even packing, and to the elimination of cracks in packed beds. Note that in zonal

synthesis rotors, the beds are packed through the upper metal shaft during rotation, with a vacuum on the lower edge line. After packing, the upper Teflon® shaft is inserted, and the upper seal assembled. Organic solid phase synthesis supports generally float in pure dichloromethane. If excessive packing is a problem, floating the bed against the center frit during each coupling cycle would be advantageous. Regardless, bed floating does not affect density-stabilized fluid flow.

Hybrid Synthesis Systems:

Before discussing results obtained in the rotor described, it should be noted that hybrid systems are feasible which combine essential properties of all of the previous four systems. A zonal synthesis rotor may be spun with its axis horizontal, and be as effective as when spun in an axis-vertical position, or it may be arranged so that the axis of rotation may be changed. If the rotor is rotated slowly with its axis horizontal, the bed may be resuspended, and kept resuspended. Resuspension may be by slow rotation, oscillation, or by shaking. If the rotational velocity is then increased, the particulate support is sedimented into a packed bed, and the bed volume can be exchanged by density-controlled zonal flow. The rotor may also be designed so that resuspension occurs with the axis vertical, using flowing gas or by mechanical shaking. These options may prove useful in much larger systems.

WORKSTATION CONTROL OF THE SYSTEM

A workstation is used to program the entire system, and to acquire, process, and display data. Programming of runs, and display of processes in progress is done through the screen shown in Figure 10 which duplicates valve positions, indicates the parameters for each step, and displays results in progress. The upper right hand corner buttons control choice between programming mode, simulation of a program, and actual synthesis. In the programming mode the values for rotor speed are set using a slider, the positions of each valve set with a cursor (and the composition of the reagent chosen automatically displayed), absorbance measurements are enabled, and the parameters to control the length of each step chosen. These include choice between time, specific absorbance measurements, density measurements, or the weight of effluent from the system. Once the parameters for a given step are chosen, they are stored, and the operator then continues on to the next step. Once a complete program is written, it may be reviewed either a step at a time, or at a preset rate to simulate a run. In the run mode, the system is completely automatic. A typical synthesis cycle plotted in terms of time and density of the reagents used is shown in Figure 11. When the sequence of reagents is in order of decreasing density, flow is in through the rotor center. When in order of increasing density, it is in from the edge. The complete prototype system is shown in Figure 12.

Synthesis of Poly T:

Using the 68 ml insert, a T12 phosphodiester polymer was synthesized with an average stepwise coupling efficiency of 97.2%, while a T11 phosphorothioate was synthesized with an average stepwise coupling efficiency of 99% in collaboration with Dr. Henri Sasmore of Isis Pharmaceuticals (the details will be published elsewhere). Over 30 additional syntheses of proprietary compounds have also been made. Our general conclusion is that there is a crossover point below which column, suspended bed techniques, or centrifugal methods are equally as good, while above that point, which is probably in the range of a bed volume of 100 ml, centrifugal systems are superior. While centrifugal systems of higher speed might prove to be even more efficient than small columns, cost considerations probably do not justify the added mechanical complexity for small scale synthesis.

OPTIMIZATION STUDIES

The high cost of doing complete large-scale syntheses precludes extensive "cut and try" experiments. Rather, methods must be found for dissecting syntheses, and optimizing each step. This requires several types of data we are only now beginning to be able to obtain, some of which is described here.

The efficiency of replacement of one solution by another in a porous support in a centrifugal field depends on the efficiency of centrifugal stabilization, on the diffusion coefficients of the solvents and solutes involved, on the porous nature of the support, on flow rate, on the density difference between succeeding solutions, and on a number of other factors including any adsorption of solutes to the support or to the growing oligo chains. All of these factors tend to widen the boundaries between reagents, and to increase the volume required to change completely one reagent for another.

The densities of reagents may, in many cases, be adjusted by changes in solvents. For example, a small amount of methylene chloride may be added to an acetonitrile solution. In the reagent schedule shown in Figure 11, the minimum density difference is between acetonitrile and acetonitrile plus synthon, and is approximately 0.06 g/mL. It is of interest to know whether this small density difference can stabilize flow, and what volumes are included in the widening fronts. Were there no such broadening, one could run reagents through as narrow zones, greatly decreasing reagent consumption.

Initially we would like to separate the study of solvent flow from that of solutes since the latter may be retarded because of molecular mass differences or adsorption. Fortunately dichloromethane (DCM) and acetonitrile (ACN) have different absorption spectra in the far ultraviolet, and we are able to analyze mixtures by following absorbance at 212 nm. In the experiment shown in Figure 13 we have filled a 340 ml rotor with spent 500 A pore size CPG, which has been extensively washed with DCM and then filled with ACN. With the rotor spinning at 2,000 rpm, a solution of 5% DCM in ACN (w/w) was run into the rotor through the edge line. The density difference between these solutions is approximately 0.016 g/mL, i.e., very much less than the density difference between any two solutions actually used in synthesis. The sharp step function shown (A) is the absorbance as measured in the input line, while (B) shows the absorbance in the output line. Note the sharp rise in A, and the slow rise in B, the delay being a measure of the liquid volume in the rotor. When the rotor was full of the DCM/ACN mixture, flow was reversed, and the rotor contents pushed out from the center with pure ACN. During this experiment the output weight was continuously measured. The total absorption difference between the two solvents was measured, and the solvent mass required to produce 98% of that difference was calculated. When flow was from edge to center, 78 g of solvent (approximately 100 ml) was required, while for flow in the opposite direction, 260 g or approximately 320 ml was required. This is a worst case example since far larger density differences than those used in this experiment occur between all steps during an actual synthesis. From this we conclude that less than a third of the rotor volume is required to change solvents during centripetal flow, while almost a rotor volume is required for flow in the other direction. For most of our syntheses to date we have used three rotor volume for solvent changes - a volume we now know is far in excess of that required. Current synthesis protocols introduce all reactants from the rotor edge.

The differences in replacement volumes with flow direction appear to be due to inefficient vectoring at the rotor wall since all of the fluid in a zone or boundary flowing to the edge must flow circumferentially around the rotor edge to reach the exit lines. While the wall of this rotor is tapered, additional improvements may be made, for example in the form of a cloverleaf configuration. Note that vectoring is improved by higher centrifugal fields.

Since absorbance measurements are made over a wide range (190-800 nm) and stored, it is feasible to follow highly absorbing materials, such as synthons, using off-peak measurements at carefully selected wavelengths. This means that it will be possible to measure synthon utilization by determining how much synthon is lost from an advancing front as compared with a control study using underivatized CPG. The results can be compared with the integrated absorption of the trityl groups released during the deblocking step.

DISCUSSION

A major objective in drug discovery and development is to find agents that react with specific

molecular targets. The targets are almost always biopolymers synthesized in vivo from a limited set of monomers including amino acids, nucleotides, and, to a lesser extent, lipids and sugars. The unique properties of these natural biopolymers derive from the order in which these monomers are assembled.

Given the extraordinary interactive specificity achieved in nature by assembling a limited set of monomers, it is natural that pharmacologists should try to mimic nature and explore ordered polymers as drugs in a systematic manner, and to produce new modifications of them which have not previously existed in nature. These include new and modified peptides, antisense oligonucleotides (1) with modified backbones, ribozymes, and a variety of interactive polymers which have been called aptamers, diversomers, and nexamers. In the past, most synthetic drugs have been synthesized as single entities by solution phase chemistry, and have, with the exception of a few peptide hormones, not been assembled in order from a set of monomers.

While short oligonucleotides and peptides have been synthesized by solution phase chemistry, and while these may ultimately be combined by fragment assembly for production purposes, it appears that for the near future commercial large-scale production will be done by solid phase synthesis. If one or more of the antisense or other sequence-specific drugs now under development come into general use as pharmaceuticals, there will be a requirement for a new class of larger scale production synthesizers which are programmable, which can control and schedule the flow of a relatively large number of different reagents, which are fully instrumented to facilitate optimization of synthesis protocols, which are based on well understood principles, but which do not now exist.

The system described here is the first step in the development of a new species of large scale systems in which all components can be readily scaled up. The requirements of incremental scale up account for our attention to a new class of valve which can be made at almost any scale, to the use of standard analytical instrumentation and use of balances to measure flow, to the use of centrifugal force to stabilize fluid movement, and to the use of the hollow-bowl zonal centrifuge configuration to provide a large bed volume with a short fluid path length. By using similar or identical materials, components, and/or basic designs at each stage in scale up, and similar chemistry, validation for the FDA is greatly simplified.

Given the high cost of the products to be made, and the fact that the value of the product in a single synthesis may exceed the cost of the synthesizer, we have felt it important to instrument the system as completely as possible, to make it easily programmable, highly reliable, and to record as much pertinent data during each run as may be useful. We have also felt it important to build very flexible instruments which can be adapted to the synthesis of a wide variety of different ordered-sequence drugs based on different chemistries.

Centrifugal synthesis provides a new and unique approach to the large scale production of biopolymers. The upper limits of this technology remain to be explored both theoretically and experimentally.

ACKNOWLEDGMENT

The development of the valve system described here was supported by an NIH SBIR Phase I Grant, No. 1 R43 GM 47757-01 to N.G. Anderson.

REFERENCES

1. Riordan, M.L., and Martin, J.C. (1991), Nature 350: 442-3.
2. Bonora, G.M., Biancotti, G., Mattini, M., and Scremin, C.L. (1993), Nucleic Acid Res. 21: 1213-17.
3. Carruthers, M.H. (1989), in Antisense Inhibitors of Gene Expression. Cohen, J.S. ed. CRC

Press, Boca Raton, FL, pp. 7-24.
4. Gait, M.J. (1984), Oligonucleotide Synthesis. IRL Press, Oxford..
5. Haller, W., (1983), in Solid Phase Biochemistry, W.H. Scouten, ed. John Wiley Sons, NY, pp 535-597.
6. Anderson, N.G., ed. (1966) The Development of Zonal Centrifuges. Natl. Cancer Inst. Monograph No. 21.
7. Anderson, N.G., Waters, D.A., Nunley, C.E., Gibson, R.F., Schilling, R.M., Denny, E.C., Cline, G.B., Babelay, E.F., and Perardi, T.E. (1969) Analytical Biochem. 32: 460-494.